Brassinosteroid Enhances Cold Stress Tolerance of Washington Navel Orange (*Citrus sinensis* L.) Fruit by Regulating Antioxidant Enzymes During Storage

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Summary

The effect of brassinosteroid (BR) on chilling injury of Washington Navel orange (*Citrus sinensis* L.) fruit was investigated. BR at the concentrations of 0.75 and 1.5 ppm effectively reduced chilling injury of Washington Navel orange fruit during five months storage at 3°C, and BR at 1.5 ppm showed the best effect. BR treatment also reduced the lipid peroxidaion and peroxide hydrogen content of peel and pulp during storage. Results of physiological response in orange fruit showed that BR induced the activity of antioxidant enzymes including catalase and peroxidase. These results indicate that the elicitation of an antioxidant response in orange fruit by BR may be associated with chilling injury alleviation. Moreover, BR maintained the orange quality by decrease of lipid peroxidation and peroxide hydrogen content. The present study is the first evidence that BR enhances orange fruit tolerance to cold stress and therefore fruit quality.

Key words

antioxidant, bassinosteroid, orange

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Introduction

Chilling injury (CI) is a primary postharvest problem for orange (*Citrus sinensis* L.) and many other horticulture crops during storage (Wang, 1993; Manners et al., 2003; Zhang and Tian, 2010). Washington Navel orange fruits are susceptible to CI during storage below 5°C (Syvertsen, 1982), and the main CI symptoms are surface pitting, browning, discoloration and decay (Schirra et al., 2005; Gualanduzzi, 2009). Several promising methods have been developed to alleviate CI symptoms of orange fruit. These include postharvest physical treatments with UV-C (Odriozola-Serrano et al., 2007; Slaughter et al., 2008), modified atmosphere packaging (Mannerset al., 2003), temperature conditioning (Obenland, et al., 2012), and chemical treatments with plant growth regulators (Montesinos-Herrero and Palou, 2010; Schirra et al., 2005; Skog and Chu, 2001).

Brassinosteroids are considered to be a class of plant polyhydroxysteroids and have been recognized as a new kind of phytohormones that play an essential role in plant development (Bishop and Koncz, 2002). Extensive studies have revealed that brassinosteroids are essential for plant growth and development since they regulate a range of physiological processes, such as stem elongation, root growth, vascular differentiation, leaf epinasty and reproduction (Fujioka and Yokota, 2003; Kim and Wang, 2010; Sasse, 2003). The potential of brassinosteroids for enhancing chilling resistance of plant cells has also been evaluated. He et al. (1991) reported that brassinolide improved the greening of etiolated leaves at lower temperatures in light and promoted the growth recovery of maize seedlings following chilling treatment. Xia et al. (2009) studied the brassinosteroids levels in cucumber (*Cucumis sativus*) through a chemical genetics approach and found that BR levels were positively correlated with the tolerance to cold stresses. Recently, abscisic acid has been shown to be involved in brassinosteroids-induced chilling tolerance in suspension-cultured cells from *Chorispora bungeana* (Liu et al., 2011). However, to the best of our knowledge, some information is available on the effects of brassinosteroids applied after harvest on CI of horticultural crops such as tomato (Aghdam, 2012). These results were consistent with Zhu et al. (2010), who found that BR significantly delayed jujube fruit senescence and maintained fruit quality.

The objectives of this study were to evaluate the effects of BR on chilling injury, lipid peroxidaion content, peroxide hydrogen content, and the induction of antioxidant enzymes, such as catalase (CAT) and peroxidase (POD), in Washington Navel orange (*Citrus sinensis* L.) fruit during storage at 3°C.

Materials and methods

Washington Navel orange fruit and treatment

Washington Navel orange (*Citrus sinensis* L.) fruits were harvested at commercial maturity from a commercial orchard in Kerman, Iran, and transported to the laboratory on the same day. Orange fruits without wounds or rot were selected based on uniformity of size and absence of physical injury or disease. The harvested fruits were disinfected with 1% sodium hypochlorite (v/v) for 2 min, washed, and dried in air. Subsequently, they were randomly divided into three groups. Two of the groups were immersed in aqueous solution containing, respectively, 0.75, and 1.5 ppm of BR for 5 min, based on our preliminary experiments, and the third group was immersed in distilled water for 5 min and served as a control. All fruits were enclosed in plastic boxes with polyethylene film bags to maintain the relative humidity at about 95% and stored at 3°C.

Evaluation of chilling injury (CI)

The symptoms of CI include surface pitting and browning. The CI index was determined according to the method described by Obenland et al. (2009). Grade levels were classified as follows: grade 0, the orange fruits are unaffected; grade 1, less than 25% of the fruit area shows CI symptoms in the peel; grade 2, 25–50% of the fruit area shows CI symptoms; and grade 3, more than 50% of the fruit area shows CI symptoms.

The CI index is calculated using the following formula: CI index (%) = (CI Grade ×number of fruit at this level)/(highest level × total fruit number) × 100. Three replicates for each treatment were performed, and each replicate contained 25 fruits.

Measurement of lipid peroxidation content

Lipid peroxidaion content was determined and expressed as malondialdehyde (MDA) equivalents, according to the method of Rajinder et al. (1981), with slight modifications. Pulp and peel tissue (4.0 g) from orange fruit were homogenized with 20 mL of 10% trichloroacetic acid and then centrifuged for 10 min at 5000 × g. One milliliter of the supernatant was mixed with 3 mL of 0.5% thiobarbituric acid (TBA) dissolved previously in 10% trichloroacetic acid. The reaction mixture solution was heat-treated for 20 min at 95°C, quickly cooled, and then centrifuged for 10 min at 10,000 × g to clarify precipitation. Absorbance at 532 nm was measured and subtracted from the nonspecific absorbance at 600 nm. The amount of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as mM g^{-1} of fresh weight (FW).

Assay of antioxidant enzyme activity

Pulp and peel samples were obtained from orange fruits at each time point during the storage at 3°C. Pulp and peel tissue samples (4 g) with 0.1 g PVPP were homogenized in 10 mL of ice-cold PBS (25 mM) containing 1 mM EDTA. The homogenate was centrifuged at 12,000 × g for 20 min at 4°C, and the resulting supernatant was collected for the enzyme assay (Egley et al., 1983).

Catalase (CAT) and peroxidase (POD) activities were analyzed according to Xing et al. (2011) with a slight modification.

The reaction mixture consisted of 2 mL sodium phosphate buffer (50 mM, pH 7.0), 0.5 mL H_2O_2 (40 mM) and 0.5 mL enzyme extract. The decomposition of H_2O_2 was measured by the decline in absorbance (A) at 240 nm. CAT specific activity was expressed as U·kg^{-1} of FW, where U = ΔA at 240 nm per s. For POD determination, 0.5 mL enzyme extract was incubated in 2 mL buffered substrate (100 mM sodium phosphate, pH 6.4 and 8 mM guaiacol) for 5 min at 30°C and the increasing absorbance measured at 460 nm every 30 s for 120 s after adding 1 mL of H_2O_2 (24 mM). POD and CAT activity was expressed as U.mg $protein^{-1}$, where U = ΔA at 470 nm per s.

Hydrogen peroxide assay

The assay for H_2O_2 content was carried out by the procedure described by Prassad (1996). Fresh tissues (2 g) were homogenized with 10 ml of acetone at 0°C. After centrifugation

for 15 min at 6000 g at 4°C, the supernatant phase was collected. The supernatant (1 m1) was mixed with 0.1 ml of 5% titanium sulphate and 0.2 ml ammonia, and then centrifuged for 10 min at 6000 g and 4°C. The pellets were dissolved in 3 ml of 10% (v/v) H_2SO_4 and centrifuged for 10 min at 5000g. Absorbance of the supernatant phase was measured at 410 nm. H_2O_2content was calculated using H_2O_2 as a standard and then expressed as µg/g on fresh weight basis (Prassad,1996).

Data analysis

All statistical analyses were performed with SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Data at each time point were analyzed by one-way ANOVA, and mean separations were performed by Duncan's new multiple range test. Differences at $P < 0.05$ were considered significant. Each treatment consisted of three replicates and the experiment was repeated six times.

Results

Effect of BR on CI of orange fruit

The effect of BR on CI of orange fruit during cold storage is shown in Fig. 1. In the untreated control orange fruit, CI symptoms (surface pitting and browning as shown in Fig. 1) occurred one month after storage, and the CI index was as high as 39.1% five months after storage in control oranges (Fig. 1). In orange fruit treated with BR, CI symptoms occurred 45 days after storage (DAS).

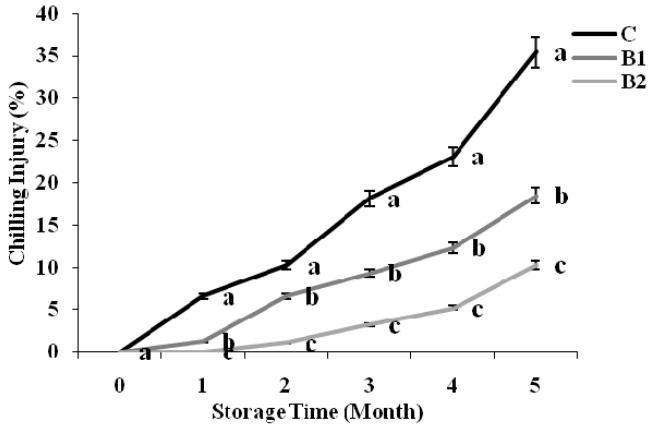

Figure 1. Effect of brassinosteroid (BR) on chilling injury of Washington Navel orange (*Citrus sinensis* L.) fruit during storage under low temperature. (C: control fruit; B1: brassinosteroid 0.75 ppm; B2: brassinosteroid 1.5 ppm). Note: Means followed by same letter are not significantly different at P<0.05 according to Duncan's multiple range test.

Moreover, the CI index in BR-treated fruit was significantly lower compared to the index in control fruit. Among all the treated fruit, BR at 1.5 ppm was the most effective in alleviating CI.

Lipid peroxidation and H_2O_2 content

Lipid peroxidaion content has been used as direct indicator of membrane injury, which is often associated with CI. As shown in Fig. 2A and B, a continuous increase in lipid peroxidaion

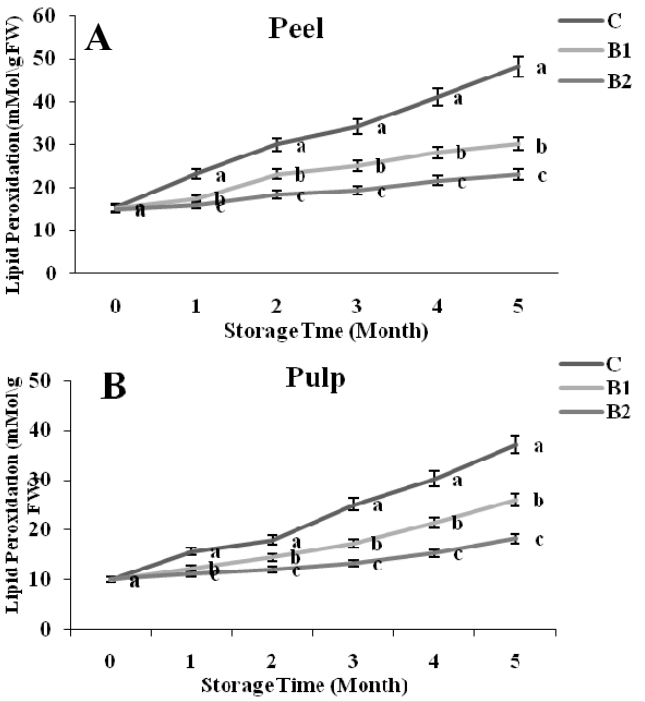

Figure 2. Effect of brassinosteroid (BR) on peel (A) and pulp (B) lipid peroxidation of Washington Navel orange (*Citrus sinensis* L.) fruit during storage under low temperature. (C: control fruit; B1: brassinosteroid 0.75 ppm; B2: brassinosteroid 1.5 ppm). Note: Means followed by same letter are not significantly different at P<0.05 according to Duncan's multiple range test.

content was observed, both in control and in the treated orange fruits stored at 3°C, although the application of BR to oranges significantly delayed the increase of lipid peroxidaion during storage. At the end of the storage period (Day 150), the lipid peroxidaion content of samples treated with BR at 1.5 ppm was significantly lower compared to the lipid peroxidation of control samples. This pattern was observed both in peel and pulp of orange fruits (Fig. 2A and B).

Changes in H_2O_2 content of orange fruit are presented in Fig. 3A and B. The initial H_2O_2 content was low (10.1 – 12.06 µg.g FW^{-1}) in peel and pulp of orange fruits. In general, H_2O_2 content increased as storage time increased. At the end of the storage period (150 DAS), the H_2O_2 content of BR-treated samples and control samples was (17.2 and 34 µg·g FW^{-1}, in the flesh, and 19.7 and 41.2 µg.g FW^{-1} in the tissue) respectively (Fig. 3A and B). The increase of H_2O_2 content in the control groups was much higher than that in the BR-treated oranges. H_2O_2 content, is as an indicator of membrane integrity (Xu et al., 2009), and our results show that membrane integrity was maintained as a result of BR treatment.

Effect of BR on induction of antioxidant enzymes

As illustrated in Fig. 4 and 5, BR treatments at 0.75, and 1.5 ppm (especially 1.5 ppm) induced the activity of CAT and POD enzymes in orange fruit stored at 3°C. CAT and POD activity

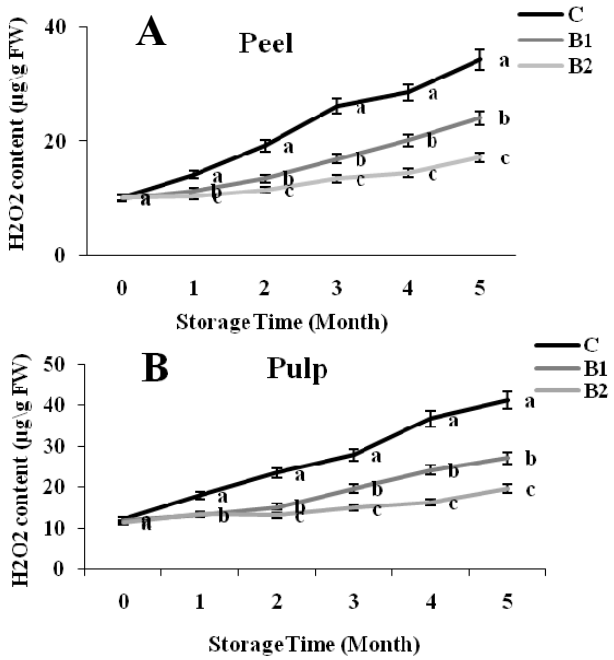

Figure 3. Effect of brassinosteroid (BR) on peel (A) and pulp (B) H_2O_2 content of Washington Navel orange (*Citrus sinensis* L.) fruit during storage under low temperature. (C: control fruit; B1: brassinosteroid 0.75 ppm; B2: brassinosteroid 1.5 ppm). Note: Means followed by same letter are not significantly different at P<0.05 according to Duncan's multiple range test.

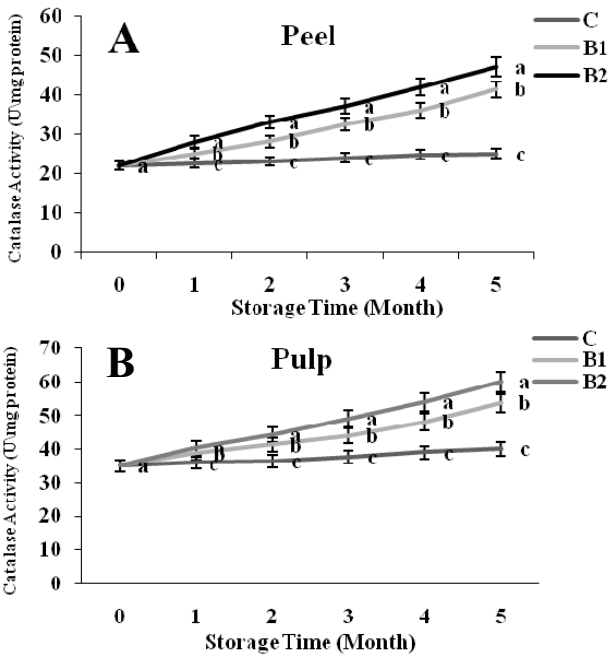

Figure 4. Effect of brassinosteroid (BR) on peel (A) and pulp (B) catalase activity of Washington Navel orange (*Citrus sinensis* L.) fruit during storage under low temperature. (C: control fruit; B1: brassinosteroid 0.75 ppm; B2: brassinosteroid 1.5 ppm). Note: Means followed by same letter are not significantly different at P<0.05 according to Duncan's multiple range test.

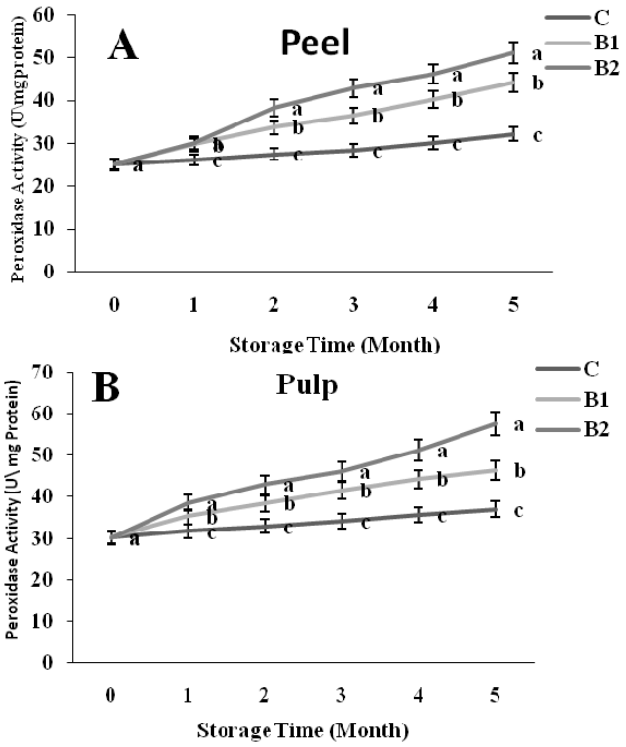

Figure 5. Effect of brassinosteroid (BR) on peel (A) and pulp (B) peroxidase activity of Washington Navel orange (*Citrus sinensis* L.) fruit during storage under low temperature. (C: control fruit; B1: brassinosteroid 0.75 ppm; B2: brassinosteroid 1.5 ppm). Note: Means followed by same letter are not significantly different at P<0.05 according to Duncan's multiple range test.

showed a similar pattern during storage in both peel and pulp (Fig. 4 and 5). In control and BR treatments activity of CAT and POD enzymes peaked at the end of the storage period (150 DAS). Both enzymes in oranges treated with BR at 1.5 ppm showed higher levels compared to controls during the entire storage period. All BR treatments induced a higher CAT and POD activity than that in control fruits (Fig. 4 and 5).

Discussion

Chilling injury (CI) is a major factor in reducing the quality and limiting the storage time of subtropical fruits, including orange. To prevent CI development and extend shelf life, a number of strategies, including physical and chemical treatments, have been evaluated (Aghdam, 2012; Obenland et al., 2009; Obenland et al., 2008; Palou et al., 2010; Schirra and D'hallewin, 1997; Smilanick, 2011). In the present study, the plant hormone BR was applied and the results indicated that BR significantly reduces CI of orange fruits during storage at 3°C (Fig. 1). Our finding was consistent with previous reports that exogenous application of BR is effective in protecting seedlings of rice (Fujii and Saka, 2001), maize and cucumber (Bajguz and Hayat, 2009) against cold stress. However, to the best of our knowledge, this is the first report that BR has been shown to have beneficial effects against CI of postharvest orange fruits. CI occurrence is

often accompanied by oxidative damage, which can be followed through lipid peroxidaion content, since it is a final product of lipid peroxidation (Xu et al., 2009). In this study, there was a continuous increase in peel and pulp lipid peroxidaion content in all fruits, but the application of BR significantly delayed the increase of lipid peroxidaion (Fig. 2A and B). Moreover, the change in membrane permeability (revealed by H_2O_2 content) showed trends similar to lipid peroxidaion content; that is, peel and pulp H_2O_2 content increased with storage duration, but BR markedly delayed the increase (Fig. 3A and B). BR has been considered to be involved in a network of interacting signal transduction pathways, which regulate defense responses to abiotic stress (Villiers et al., 2012). Divi et al. (2010) demonstrated that BR-mediated stress tolerance in arabidopsis shows interactions with abscisic acid, ethylene and salicylic acid pathways. The mechanism by which BR induced cold resistance in orange was investigated in this study. When horticultural crops are exposed to severe abiotic stresses, including cold stress, large amounts of intracellular ROS (reactive oxygen species) are generated (Gualanduzzi et al., 2009; Wagstaff et al., 2010). The detoxification of ROS is dependent on antioxidant enzymes such as CAT and POD (Aghdam, 2012; Ding et al., 2007). The increase in these enzymes' activity contributes to the adaptation of plants to cold stress and ameliorates oxidative damage such as lipid peroxidation (lipid peroxidaion increase as indicator) and H_2O_2 content (Aghdam, 2012; Huang and Guo, 2005). In the present study, we found that the activity of the two enzymes in orange was induced by BR treatment during the storage at 3°C (Fig. 3 A and B).

Similar results have been reported in previous studies. Verma et al. (2012) demonstrated that exogenous BR was effective in promoting *in vitro* growth of groundnut (*Arachis hypogaea*) by elicitation of CAT, POD, APX (ascorbate peroxidase) and polyphenol oxidase. Zhang et al. (2010) reported that BR enhanced the activity of CAT, APX, and SOD (superoxide dismutase) in maize leaves, and apoplastic H_2O_2 in BR-induced antioxidant defense. We suggest that the BR induction of antioxidant enzyme activity in orange fruits may be a key factor in lowering oxidative damage caused by cold stress, thus improving the cold tolerance and alleviating CI of orange stored at 3°C.

Conclusion

In conclusion, application of BR reduced CI of oranges stored at 3 °C and maintained oranges quality as well. The chilling injury, lipid peroxidaion, and peroxide hydrogen were significantly reduced by brassinosteroid treatment especially at 1.5 ppm. BR treatment induced cold resistance may be due to stimulation of antioxidant enzymes and protection against membrane oxidative damage, decreased lipid peroxidation and H_2O_2 content in orange fruits. These results may have implications for the use of BR in managing postharvest CI of other subtropical fruits stored at low temperatures.

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